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Estrogen is a key hormonal regulator of mammary epithelial cell growth. Thus, understanding the mechanisms of estrogen-mediated growth regulation is key to understanding to understanding growth disregulation in breast cancer. Current information indicates that estrogen regulates cell growth by activating the Raf-1/Mek/MAP kinase pathway and upregulating Cyclin D1. Genetic and biochemical analysis of the Cdc37 protein has strongly implicated it in regulation of the Raf-1 Map kinase pathway, cylin/cdk association, and steroid receptor function. Here we have examined the role of $p50^{cdc37}$ and its Hsp90 chaperone partner in Raf/Mek/MAP kinase signaling and in estrogen receptor function. We have found that $p50^{cdc37}$ is the primary determinant of Hsp90 recruitment to Raf-1. Overexpression of a $p50^{cdc37}$ mutant which is unable to recruit Hsp90 into the Raf-1 complex inhibited Raf-1 and MAPK activation by growth factors and estrogen. The dominant negative Cdc37 also inhibited transactivation of an estrogen responsive reporter gene in the human MCF-7 breast cancer cell line. We have found that coexpression of wild-type $p50^{cdc37}$ with Raf-1 results in robust and dose-dependent activation of Raf-1 in Sf9 cells. Thus, $p50^{cdc37}$ plays a crucial role in Raf-1 activation and MAPK pathway signaling in general and in estrogen action in particular.

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INTRODUCTION

Estrogens are key regulators of mammary cell proliferation. Some human breast cancers even remain estrogen dependent for growth(43). It is generally thought that estrogen independent breast cancers have evolved mechanisms to circumvent estrogen regulation(29, 99). Thus, understanding the mechanisms for of estrogen mediated growth regulation in mammary tissue is essential to understanding of breast carcinoma. Previous work on estrogen dependent growth regulation has implicated estrogen regulation of the Raf-MAP kinase pathway and of cyclin D1 expression as being critical for the mitogenic effects of estrogen(7, 50). It is likely that MAP kinase activation is directly responsible for the regulation of cyclin D1 expression(41, 64, 99). Moreover, cyclin D1 is over expressed at high percentage of breast cancers indicating that this may be a critical in breast oncogenesis(80, 99). Here we have investigated the role of the CDC37 gene in the regulation of cellular responses to estrogen and in the MAP kinase pathway.

The mitogen-activated protein kinase (MAPK) phosphorylation cascade, composed of Raf kinase, Mek (MAPK kinase), and Erk(MAPK) itself, relays proliferative and differentiative signals from the plasma membrane to the transcriptional and cell-cycle progression machinery (49). Although it is established that ras-GTP is required to tether Raf-1 to the plasma membrane (reviewed in 2), the subsequent events that lead to Raf-1 activation are poorly understood. The major reasons for this are first, that only a small fraction (~3 %) of the total Raf-1 cytoplasmic pool needs to become activated for effective signaling(27) and, second, that the entire process of Raf-1 plasma membrane recruitment and activation is rapid and transient (for review, see Marais and Marshall (47) and Morrison and Cutler (56)). Thus, identification of both crucial intermediates and the causative relationships in Raf-1 activation has been difficult. However, it is clear that the N-terminal domain of Raf-1 acts to repress the activity of the C-terminal kinase domain and its deletion results in constitutive activation of the kinase (28, 85). Phosphorylation of Raf-1 and association with other proteins in response to receptor activation most likely leads to a conformational change in Raf-1 that relieves this repression (47, 56).

Raf-1 fractionated from various cell types exists in large (300- to 500-kDa) multiprotein complexes (95). Known Raf-1 associated proteins include 14-3-3, Hsp90, and pp50, a 50-kDa Hsp90 associated protein (56, 95). 14-3-3 is required for Raf-1 function, but probably is not directly involved in the Raf-1 activation process (47, 53, 55). The function of the pp50/Hsp90 complex in Raf-1 activation has yet to be addressed. pp50 had previously been widely found in Hsp90-containing kinase complexes, notably involving v-Src (reviewed in 6) and with both cytoplasmic and membrane localized Raf-1 (84, 95). Hsp90 associated pp50 has recently been identified immunologically and by peptide mapping to be the 50 kDa gene product of the mammalian cdc37 homologue $p50^{cdc37}$ (66).

Cdc37 was originally identified in yeast as a cell cycle mutant that gives a G1 cell cycle arrest phenotype (72). Cutforth and Rubin (11), subsequently isolated an allele of Drosophila cdc37 (Dcdc37) that functioned as a dominant enhancer of the *sevenless* phenotype in the Drosophila eye. This work demonstrated that Dcdc37 functions in insects in the same signaling pathway as does Drosophila Raf. Vertebrate Cdc37 was first cloned from chick (25, 31) and subsequently from mammals (24, 40, 62, 66, 86). The structure of Cdc37 reveals no significant homologies to proteins of known function. The yeast protein is homologous to mammalian and Drosophila Cdc37 through only the first 30 amino acids and diverges significantly thereafter. Despite this limited homology, the Drosophila Cdc37 will complement the yeast gene(11). The cell cycle phenotype of *cdc37* appears to be due to a diminished capacity of G1 cyclins and the cyclin dependent kinase Cdc28p to associate(23). Subsequent work by ourselves and others has found that mammalian $p50^{cdc37}$ interacts with Cdk4 and accumulates Hsp90 to it (13, 24, 40, 86). Though $p50^{cdc37}$ has been found to interact with diverse kinase families, its interactions are selective in that, for instance, among cyclin dependent kinases, it interacts only with Ckd4 (13, 32, 86). Thus, from genetic studies, Cdc37 appears to operate in both the cell cycle and the Ras/Raf/MAPK pathway in close cooperation with its Hsp90 chaperone partner(32).

Hsp90 is an abundant and highly conserved protein (70) that is essential in yeast and Drosophila (4, 11). Unlike the Hsp70 and Hsp60 chaperones, Hsp90 appears to have substrate

specific folding activity (33, 58, 70). It has been best characterized for its essential role in steroid hormone receptor signaling where it interacts with and modulates receptor function through a dynamic and regulated series of interactions with a defined set of chaperone cofactors (70, 83). Hsp90's conformation and activity has been proposed to be regulated by nucleotide binding and its associations and activity can be inhibited by geldanamycin, an Hsp90 specific antibiotic, which competes for ATP binding to Hsp90 (26, 71). It has been further proposed that $p50^{cdc37}$ may serve to target Hsp90 to a subset of protein kinases and thereby help them achieve an active conformation(32, 69). However, the yeast Cdc37p by itself has been shown to have chaperone activity in vitro (38).

The available mammalian association data, although not informative about the functional significance of Raf-1 association with Hsp90 and $p50^{cdc37}$, nevertheless, are complemented by genetic evidence from Drosophila. Cutforth and Rubin (11) found that Hsp90 mutations enhance the *sevenless* phenotype in the Drosophila eye as does Dcdc37 and thus also functions in the MAPK pathway. Subsequently, van der Straten et al. (93) identified Hsp90 alleles that suppress the multiple R7 phenotype caused by the constitutive high level activation of a membrane-targeted D-Raf kinase domain (Raf^{torY9}). In fact, the two Hsp90 point mutations recovered in this screen, were the strongest dominant suppressors of the multiple R7 photoreceptor-cell phenotype caused by the, Ras-independent, activated Torso RTK/Raf chimeric protein. Importantly, the mutant Hsp90 proteins identified in these genetic screens exhibited reduced binding to D-Raf-1 and correlated with diminished Raf kinase activity (93). Thus, neither deletion of the N-terminal suppression domain, nor membrane-anchoring allow Raf^{torY9} to overcome the requirement for Hsp90 association.

Here, we have investigated the biochemical role of $p50^{cdc37}$ and its partner, Hsp90, during Raf-1 activation and signaling to Mek and Erk. We have found that $p50^{cdc37}$ and Hsp90 each interact directly with Raf-1, but that $p50^{cdc37}$ is the primary determinant of the assembly of heterotrimeric complex . Disruption of the Raf-1/$p50^{cdc37}$/Hsp90 ternary complex with the Hsp90 inhibitor, geldanamycin, or with a dominant negative $p50^{cdc37}$ inhibits Raf-1 activity. Serum

stimulation promotes Raf-1/p50^{cdc37}/Hsp90 complex formation and coexpression of p50^{cdc37} with Raf-1 in insect cells is sufficient to activate Raf-1. Moreover, p50^{cdc37} synergizes with Src for Raf-1 activation. In addtion, we have found that dominant negative p50^{cdc37} inhibits the ability of estrogen to active that MAP kinase pathway and to activate a reporter gene in MCF-7 mammary carcinoma cells. Our data coupled with the aforementioned genetic studies, indicate that p50^{cdc37} and Hsp90 are critical components of the MAPK cascade and of the Raf-1 activation complex, in general and are critical for the estrogen response in particular.

MATERIALS AND METHODS

Cell culture and transfections. Cos-1 cells , MCF-7 cells, and human embryonic kidney 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.1 μg/ml penicillin and streptomycin. Experiments with MCF-7 cells were performed in phenol red free media. Freshly plated cells were transfected at 70-80% confluence with a total of 7.5 μg DNAs per 100 mm dish using the lipofectamine (Gibco-BRL) method. In experiments requiring replicate transfected cultures, cells were split 24 hours post start of transfection into appropriate smaller size dishes so that 20 to 24 hours later cultures would have achieved confluence. At this point, cells were serum starved for an additional 16-18 hours. For stimulations, serum (at 20%) or epidermal growth factor (EGF; 100 ng/ml), was directly added for 5 more minutes before cells were lysed. A 2 mg/ml stock solution of geldanamycin (GA) in DMSO, or DMSO was diluted 1:1000 in the culture media for the times indicated before cells were either lysed directly or serum stimulated. Solubilized cell extracts were then quantitated for protein content by the Bradford assay and analyzed by direct Western or by protein purification using antibodies or, for overexpressed GST-fusion proteins, by glutathione (GSH) sepharose chromatography, followed by SDS-PAGE and immunoblotting. Baculovirus infection and culture of Sf9 cells was performed essentially as described in Morrison (54). Otherwise indicated, all baculoviruses were infected at comparable levels of multiplicity of infection (m.o.i.). For luciferase reporter assays with the ERE luciferase construct(36) and the Elk-1 Path Detect sytstem (Stratagene), dual luciferase assay was carried out following the manufacturer's recommendation (Promega). All transfection experiments were performed in duplicate and results were normalized to the expression of the renilla luciferase transfection control.

Antibody reagents. The anti-$p50^{cdc37}$ antibodies were raised in rabbits against the chick (pNG13 clone, Grammatikakis et al., 1995(25)) or the human GST-$p50^{cdc37}$ proteins. Anti-epitope tag antibodies were obtained from Boehringer (12CA5/anti-HA; 9E10/anti-MYC), or from Kodak (M5/ anti-FLAG). Santa-Cruz was the supplier for additional antibodies, including ones

against Raf-1 (C-12) and GST (Z-5). Monoclonals against Raf-1 and $p50^{cdc37}$, used in the experiment described in figure 1B, were purchased from Transduction Laboratories. Anti-active MAPK polyclonal (V6671) was obtained from Promega and antibodies directed against Hsp90 (SPA-830 and SPA-771) were obtained from Stressgen.

Cloning and constructs. For eukaryotic expression, the complete open reading frame for the human $p50^{cdc37}$ cDNA was subcloned by PCR into the EcoRI sites of pMT3 or pSG5 vectors and in frame with an N-terminal hemagluttinin (HA) or FLAG, respectively, peptide epitope. Similarly, GST-$p50^{cdc37}$ constructs placed by PCR into the BamHI-NotI sites of the pEBG eukaryotic (75) or pGEX2T (Pharmacia) prokaryotic expression vectors. For expression in insect (*Spodoptera frugiperda;* Sf9) cells, the entire ORF for the FLAG-$p50^{cdc37}$ fusion protein was subcloned from the pSG5 constructs into the EcoRI/NotI sites of the pFASTBAC1(Gibco/BRL) baculovirus vector. Deleted versions of the FLAG-$p50^{cdc37}$ fusion protein were produced by using appropriate enzyme digestion of the full length inserts on pSG5, followed by agarose gel electrophoresis and DNA religation and further subcloned into pFASTBAC1 using the same approach. Cloned inserts were verified by DNA sequencing. Expression plasmids for Raf-1, Mek-1, and Erk-1 used in this study have been described previously (17, 45, 57, 81).

***In vitro* synthesis of radiolabeled $p50^{cdc37}$.** Different full-length and deletion forms of $p50^{cdc37}$ were transcribed and translated *in vitro* from the pSG5 expression constructs in the presence of 20 μCi of [^{35}S] methionine (EXPRESS protein labeling mix, New England Nuclear, NEN), using the coupled rabbit reticulocyte lysate and T7 RNA polymerase system (Promega).

Metabolic labeling. Non-transfected or transfected cells 48 to 60 hours post transfection were initially incubated for 2 hours in methionine-free medium containing 2% dialyzed fetal serum and then labeled for 4 hours with [^{35}S]-methionine (NEN) in fresh medium. Cells were then lysed and equal amounts (counts per minute) of labeled lysate were immunoprecipitated, as described below for non-labeled lysates, and analyzed by SDS-PAGE and fluorography.

Immunoprecipitation and immunoblotting. Cells were harvested 48-60 hours after transfection and extracted in lysis buffer (NP-40 LB: 0.5% Nonidet P-40, 20 mM Hepes (pH 7.5), 0.1 M NaCl, 2mM EGTA, 10% glycerol, 50mM glycerophosphate, 2mm DTT) containing protease and phosphatase inhibitors (2mM sodium vanadate, 1mM NaF, 0.2 mM PMSF and 10 μg/ml each of leupeptin, aprotinin). For measuring Raf-1 kinase activity in Sf9 cells in the experiments described in figures 4,5B, and 6A, NP-40 LB was substituted with RIPA buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 10% v/v glycerol, 1% v/v NP-40, 0.1% SDS (w/w), 0.5% sodium deoxycholate, 2 mM EDTA). Cell lysates were cleared by centrifugation at 4°C for 15 min. The protein concentration was measured with a kit from Biorad and normalized for all samples in each individual total Western or immunoprecipitation experiment. Equivalent aliquots of cleared supernatants were mixed with Laemmli SDS-loading buffer (25 mm Tris , pH 6.8, 1% SDS, 2.5% β-mercaptoethanol, 0.5 mg/ml bromophenol blue, 5% glycerol), separated by SDS-PAGE and transferred to Hybond-ECL membrane (Amersham). Following preclearing, immunoprecipitation was performed, for 2 hrs at 4°C, using 0.5 μg of purified anti-FLAG , anti-c-MYC , anti-HA monoclonal or indicated purified rabbit polyclonal antisera. Immune complexes were then recovered by binding to GammaBind-Plus Sepharose (Pharmacia). Alternatively, GST-fusion proteins were purified using pre-equilibrated GSH-Sepharose (Pharmacia) as described(82). After three washes with 50 volumes lysis buffer, GSH-Sepharose bound proteins and immunocomplexes were processed for electrophoresis as above. The entire protein purification procedure was done at 4° C. Immunoblot detection was performed with specified antibodies in 5% dried milk in PBS and developed as described by the manufacturer of the enhanced chemiluminescence system (ECL; Amersham,). For reblotting, membranes were incubated in 20 mM DTT;1%SDS in PBS for 10 min at ambient temperature.

Protein purification and *in vitro* association assays. GST-fusion proteins were produced and purified by GSH-Sepharose affinity chromatography in NETN buffer (20 mM Tris, pH 8.0, 0.1M NaCl, 1 mM EDTA, 0.5% NP-40) supplemented with proteinase and phosphatase inhibitors as previously described (Smith and Johnson, 1988). Kinase-defective (KD) bacterial

$(His)_6$-Mek-1 (K97M) was similarly prepared using a kit from Qiagen. FLAG-$p50^{cdc37}$ was immunoaffinity purified by agarose-crosslinked anti-FLAG M2 monoclonal (Kodak) according to the supplier's instructions. For studying *in vitro* associations, GSH-sepharose bound GST-fusion proteins were then directly incubated with either purified or in vitro translated proteins in NETN buffer for two hours at 4°C. Bound complexes were subsequently washed 3 times in 50 volumes of prechilled NETN buffer and after SDS-PAGE they were either immunoblotted or, for ^{35}S methionine-labeled proteins, directly analyzed by fluorography.

Protein kinase assays. For kinase reactions, GSH-sepharose bound GST-fusion proteins or immunocomplexes prepared as above, were additionally washed in 50 volumes kinase buffer (25 mM Hepes , pH 7.5, 10 mM $MgCl_2$, 1 mM DTT (and $MnCl_2$ at 10mM for Raf-1 kinase assays)), and then drained and incubated for 15 min at 30°C in 30 µl fresh kinase buffer containing 20 µM ATP, 5 µCi[γ-^{32}P] ATP (NEN; 6000 Ci/mmol) and 0.5 µg recombinant kinase-defective $(His)_6$-Mek-1 (K97M), GST-Erk-1 (K71A), or 1 µg MBP (UBI, Lake Placid), substrate proteins. Assays were terminated by the addition of Laemmli SDS-loading buffer, the boiled samples were resolved by SDS-PAGE and phosphorylated substrate proteins were quantitated by phophorimager analysis and autoradiography.

RESULTS

Domains of interaction between $p50^{cdc37}$, Hsp90, and Raf-1. To characterize the precise interactions among $p50^{cdc37}$, Hsp90 and Raf-1, the coprecipitation profile of these proteins was examined in Cos-1 cells. Cos-1 cells express Raf-1, which is the principle Raf isoform (19), and both Hsp90 and $p50^{cdc37}$. In accordance with previous findings from other tissues (14, 15, 44, 95), two proteins of approximately 90 and 50-kDa coprecipitate with endogenous Raf-1 in Cos-1 cells (fig. 1A). Subsequent disruption of the complex and a second round of immunoprecipitation (IP) with anti-Hsp90 and anti-$p50^{cdc37}$ antisera indicates these two co-precipitating proteins are immunologically related to Hsp90 and $p50^{cdc37}$, respectively (Fig. 1A,

lanes 1-5). The converse experiment precipitating first with anti-p50^{cdc37} antibodies shows stoichiometric co-immunoprecipitation with Hsp90, but reveals only a faint Raf-1 band at the expected 74-kDa range (lanes 6-8). This is probably due to the fact that although a significant proportion of Raf-1 protein is bound to p50^{cdc37} and Hsp90 (44 and Grammatikakis, N., unpublished, 77, 95), only a fraction of p50^{cdc37}, which is present in excess over Raf-1 (not shown) and Hsp90 (1-2% of total cytosolic protein), is in a complex with the kinase. Our findings with both ^{35}S-methionine labeled proteins (figure 1A, lanes 6-8) and by silver staining (not shown) indicate that Hsp90 copurifies quantitatively with p50^{cdc37} and that the p50^{cdc37}/Hsp90 interaction also occurs in vitro in the absence of other proteins (81).

That the recombinant p50^{cdc37} protein indeed associates with Raf-1 is further supported by the experiments presented in figure 1B, where either overexpressed p50^{cdc37} or endogenous p50^{cdc37} was immunoprecipitated from Cos-1 cell lysates with anti-p50^{cdc37} antibody and Western blotted with anti-p50^{cdc37}, Raf-1, or Hsp90. In both situations, similarly sized 50-kDa proteins were found in complex with endogenous Raf-1 and Hsp90. p50^{cdc37}'s associations were sensitive to RIPA buffer (lane 2) and were specific, in that no Hsp90 or Raf-1 could be observed in control antibody IPs (lane 1). Conversely, anti-Raf-1 IPs, followed by Western blotting analysis identified both p50^{cdc37} and Hsp90 at lower levels, but in a reproducible manner, to copurify with endogenous Raf-1. Thus, by its size and characteristics of its interaction with Raf-1 and Hsp90, cloned p50^{cdc37} is most likely, pp50, the previously described 50-kDa Hsp90 partner present in the Raf-1 IPs along with Hsp90. Similar conclusions were reached *in vitro*, using combinations of purified Hsp90 and p50^{cdc37} proteins to reconstitute these associations (fig. 1C).

To test whether posttranslationally unmodified Raf-1 can bind to Hsp90 and p50^{cdc37}, GSH-Sepharose bound GST-Raf-1 that had been produced in *E. coli* was allowed to associate with either p50^{cdc37} or Hsp90 alone or with a mixture of the two proteins. Both purified p50^{cdc37} and Hsp90 were found to interact directly and independently with recombinant Raf-1 *in vitro* (fig. 1C, bottom panel). Notably, Hsp90's association with Raf-1 greatly increased when p50^{cdc37} was present. This result suggests that either Hsp90's association with Raf-1 is induced by a p50^{cdc37}-

mediated Raf-1 conformational change, or that, more likely, the strong association between Raf-1 and Hsp90 is mediated by $p50^{cdc37}$ acting directly to recruit Hsp90 to Raf-1. In the latter scenario, the existence of two distinct sites on Hsp90, one for associating with the $p50^{cdc37}$ and a second for directly binding to Raf-1, can be envisioned (see scheme in fig. 2D). These experiments demonstrate that recombinant $p50^{cdc37}$ and Hsp90 associate directly and stably with Raf-1 confirming the earlier identification of these proteins by immunological means (66, 77, 84, 95).

Since the catalytic C-terminal half of Raf-1 has been reported to be sufficient for interaction with pp50 (84), we tested whether recombinant $p50^{cdc37}$ binds to the same Raf-1 region. *In vitro* translated $p50^{cdc37}$ bound efficiently to immobilized GST-ΔN-Raf-1, a viral Raf form-like construct (5, 81), but not to GST alone (fig. 2A) or to the N-terminal Raf-1 regulatory domain alone (not shown). This interaction of $p50^{cdc37}$ with Raf-1 occurs via the N-terminal half of $p50^{cdc37}$ as a deletion mutant ($p50^{cdc37}\Delta C$) truncated at Met164 to half the original size, is sufficient to interact strongly with GST-ΔN-Raf-1. Interestingly, $p50^{cdc37}\Delta C$ is severely compromised in its ability to associate with Hsp90 in transfected Cos-1 cells (fig. 2B) compared with full-length $p50^{cdc37}$ which readily associates with its chaperone partner.

We then sought to determine whether this mutant might be able to disrupt the Hsp90/Raf-1 association in a dominant fashion. When $p50^{cdc37}\Delta C$ was further co-expressed in Cos-1 cells with GST-tagged Raf-1, endogenous Hsp90 association to Raf-1 was strongly inhibited in a dose-dependent manner with increasing amounts of $p50^{cdc37}\Delta C$ binding to the kinase (fig. 2C). In contrast, overexpressed wild-type $p50^{cdc37}$ not only binds to Raf-1 but also recruits Hsp90 to the complex, in agreement with the *in vitro* experiment shown in figure 1C. A likely interpretation of this observation is that overexpressed $p50^{cdc37}\Delta C$ competes with endogenous $p50^{cdc37}$ for binding to Raf-1 and that the subsequent Hsp90 association with GST-Raf-1, which largely depends on intact $p50^{cdc37}$, is prevented (fig. 2C, cf. lanes 1, 3, 5). Thus, although some direct Hsp90 binding to Raf-1 cannot be ruled out (see fig. 1C, lane 4), we conclude that the $p50^{cdc37}$/Raf-1 interaction is the primary determinant of Hsp90 accumulation into the complex (figure 2D). This result also suggests that $p50^{cdc37}\Delta C$ might interfere with the function of Hsp90 in the Raf-1

complex and potentially act as a dominant negative allele of $p50^{cdc37}$ in functional assays (described below).

Serum stimulation promotes and geldanamycin inhibits the assembly of the Raf-1/$p50^{cdc37}$/Hsp90 complex. Geldanamycin (GA), a benzoquinone ansamycin, has been established to be a specific reagent for assessing Hsp90's role in various signaling systems, including v-Src(98), Raf-1 (77, 78), and steroid nuclear receptors (35, 83) among others (reviewed in 68) . GA competitively displaces ATP and locks Hsp90 into its ADP-specific inactive conformation, disrupting a dynamic equilibrium in which unliganded steroid receptor complexes alternate among various heterocomplex intermediates (26, 35, 71, 83). GA-bound Hsp90 is then unable to form productive complexes with its steroid receptor and kinase targets, which subsequently results in their degradation upon prolonged *in vivo* GA treatment (76, 77, 79, 98). In an attempt to define the roles of $p50^{cdc37}$ and Hsp90 in Raf-1 kinase heterocomplex formation and activity, we have made use of GA to abrogate Hsp90/Raf-1 association and Raf-1 activation as has been shown by Schulte et al. (77, 78). However, in order to directly correlate Raf-1's ability to interact with $p50^{cdc37}$ and Hsp90 with its kinase activity, we have designed our experiments to assess the effects of GA on Raf-1 at a stage prior to the time when Raf-1 has been depleted from the cells due to prolonged GA treatment. In addition, to improve the detection of associated proteins, especially of $p50^{cdc37}$, which migrates closely on SDS-PAGE with immunoprecipitating antibodies, we have alternatively employed GST-fusion cDNAs of Raf-1 or $p50^{cdc37}$ transiently transfected into Cos-1 cells. Glutathione (GSH) sepharose purified GST-Raf-1 or GST- $p50^{cdc37}$ were then analyzed both for associated proteins and for kinase activity (75, 82). In addition, since Mek-1 has previously been found to stably copurify with Raf-1 (92, 95), we have also transiently expressed it as a GST-fusion protein and, similarly, analyzed its potential interactions with endogenous Raf-1, $p50^{cdc37}$ and Hsp90 (fig. 3).

Cos-1 cells were transfected with either GST-$p50^{cdc37}$, GST-Raf-1, or GST-Mek-1 and replated into three identical cultures. These cultures were serum starved overnight and two of the

replicate transfections were then stimulated with 20% serum with or without a 6 hour preincubation with GA, as indicated, while the third plate was left untreated. The resulting cellular extracts were analyzed for overall protein expression, protein association with each purified GST-protein, and for respective *in vitro* kinase activity using recombinant kinase inactive forms of Mek-1 and Erk-1 as substrates (fig. 3). Western blotting of total cell extracts revealed that expression of the transfected GST-fusion proteins was approximately three times the level of the corresponding endogenous $p50^{cdc37}$, Raf-1 and Mek proteins (not shown) and that GA treatment slightly reduced the levels of Raf-1 expression, but had no apparent effect on $p50^{cdc37}$, Hsp90, and Mek-1 steady state levels. From this experiment, the following observations can be made. Consistent with the existing literature, both transfected GST-Raf-1 and GST-Mek-1 kinase activities were induced by serum, but not after GA pretreatment (fig. 3 I). Interestingly, both $p50^{cdc37}$ and Hsp90 are clearly associated with activated Raf-1. Accordingly, serum stimulation results in small but reproducible enhancement of associations of endogenous $p50^{cdc37}$ and Hsp90 with GST-Raf-1 (fig. 3 II, lanes 1 and 2). In contrast, GA pretreatment of the serum-deprived GST-Raf-1-transfected Cos-1 cells abolished activation of Raf-1 by serum and almost entirely eliminated this association. Importantly, Raf-1 association with $p50^{cdc37}$/Hsp90 correlates closely with its activity (fig. 3 II, lanes 1 and 2). Previously, GA has been shown to decrease Raf-1 activity and expression in NIH3T3 cells by destabilizing the protein(77, 78). Note that in this experiment, using a limited exposure of GA to Cos-1 cells, GST-Raf-1 expression is only modestly reduced (fig. 3 II and III, lane 6), but both Hsp90 and $p50^{cdc37}$ associations with GST-Raf-1 are nearly abolished. Thus, disruption of the Raf-1/$p50^{cdc37}$/Hsp90 complex by GA correlates with the inhibition of Raf-1 activation by serum growth factors.

Interestingly, overexpressed GST-$p50^{cdc37}$ remained sequestered stoichiometrically with endogenous Hsp90 under all conditions, including GA pretreatment. Its respective associations with endogenous Raf-1 also showed small but reproducible serum-mediated enhancement and almost complete elimination by GA (fig. 3 II, lanes 4-6). Thus, during serum activation of Raf-1, there is a stabilization of $p50^{cdc37}$/Hsp90/Raf-1 complex formation. Only a weak associated

MAPKKK activity could be detected in GST-p50^{cdc37} pull-down/*in vitro* kinase assays after very long autoradiography exposure (not shown), consistent both with our observation that the bulk of p50^{cdc37} is not Raf-1-associated (fig. 1A) and the fact that only a small fraction of Raf-1 kinase actually becomes activated during signaling (27, 47, 56). Nevertheless, both GST- p50^{cdc37} and co-precipitating Hsp90 were found to be associated with and phosphorylated by an unidentified kinase that was absent or inactive in the GA-treated sample, in agreement with previous reports describing Hsp90 and its 50-kDa partner in v-Src and Raf-1 heterocomplexes as phosphoproteins (6, 65, 95). We do not believe this kinase to involve Raf-1 since it is apparently quite active even under serum-deprived conditions (see also the Discussion).

Following its activation in response to growth factor stimulation, Raf-1 phosphorylates and activates Mek-1. Thus, we examined the effect of GA on Mek-1 complex formation and activity. As expected, GST-Mek-1's activity was increased by serum, but not after GA pretreatment, in agreement with Schulte et al. (78). In general, we find Raf-1 binding to Mek-1 to be weaker than its interaction with p50^{cdc37} (see also anti-Raf-1 Western Blots short and long exposures in fig. 3). In contrast with Raf-1/p50^{cdc37} association, Raf-1/Mek1 association did not fluctuate upon serum stimulation and was insensitive to GA. Interestingly, we have further detected a stable complex of endogenous p50^{cdc37} with GST-Mek-1 (fig. 3) and between the endogenous p50^{cdc37} and Mek-1/-2 proteins (not shown). Strikingly, Mek-1/p50^{cdc37} complexes are unusual in that they are devoid of Hsp90, the usually constitutive p50^{cdc37} partner (figure 3). By direct comparison, the avidity of p50^{cdc37} for GST-Mek-1 was found to be much weaker than that between p50^{cdc37} and GST-Raf-1 (not shown and see anti-GST immunoblots in figure 3 for transfected GST-Mek-1 and GST-Raf-1 normalization). The p50^{cdc37}/Mek-1 complex is most pronounced in serum-deprived and GA treated cells, and is somewhat reduced following serum stimulation (fig. 3 II, lanes 7 - 9, and results not shown). Thus, Raf-1 and Mek-1 distinctly differ in their capacity to form Hsp90-associated and Hsp90-free p50^{cdc37} associations, respectively. Intriguingly, Hsp90-free p50^{cdc37} exhibits enhanced association with Mek-1 under conditions where Raf-1 is least bound to p50^{cdc37}/Hsp90 and inactive, suggesting that p50^{cdc37}'s targeting to either Mek-1 or Raf-1 kinases

might possibly depend on the activation state of the Raf-1 kinase. Consistent with this, our data, including those presented in figure 3, indicate that $p50^{cdc37}$ and Mek-1 exhibit differential binding preferences for the presumably differentially phosphorylated faster and slower migrating forms of endogenous Raf-1, respectively (denoted by arrows in anti-Raf-1 immunoblots in fig. 3, lanes 4-9).

In addition to its effects on serum activation of Raf-1, in experiments similar to the one described in figure 3, we have found that GA also inhibits Raf-1 activity driven by cotransfected Ras(Q61L), a constitutively active Ras mutant (not shown). This result indicates that inhibition of Raf-1 by GA occurs downstream of Ras in agreement with the original observations of Schulte et al. (77, 78) who found that GA had no effect on Ras levels and on Raf-1/Ras-GTP interaction. We have further observed that, as with serum induction, activated Ras potentiates Raf-1 association with the $p50^{cdc37}$/Hsp90 complex, but in the presence of GA, this association is entirely abolished although the $p50^{cdc37}$-Hsp90 association again remains unaffected. Altogether, the above results suggest that $p50^{cdc37}$-Hsp90 association with Raf-1 is required for the activation and/or maintenance of Raf-1 activity.

Activation of Raf-1 by $p50^{cdc37}$ overexpression. The Sf9 insect cell/baculovirus expression system is currently the most widely used *in vivo* system for evaluating potential Raf-1 activators (reviewed in 55). Therefore, we have used this system to further analyze the possible involvement of $p50^{cdc37}$ in the Raf-1 activation process. Baculoviruses expressing full-length $p50^{cdc37}$ and Raf-1, together, or in triple combinations with v-Src or v-Ras expressing baculoviruses (fig. 4A) were used to co-infect Sf9 cells. 48 hours post-infection, Raf-1 was immunoprecipitated from Sf9 cells in RIPA buffer and subsequently assayed for its ability to phosphorylate inactive recombinant Mek-1. Consistent with previous reports (reviewed in (55)), v-Src, and to a lesser extent, v-Ras, both activate Raf-1, an effect most prominent when both oncoproteins are coexpressed (fig. 4A, lanes 1-4). Surprisingly, $p50^{cdc37}$, a unique protein with no apparent kinase or other recognizable enzymatic domain, by itself strongly activated Raf-1 to an

extent even greater than v-Ras and almost as well as, although never exceeding, v-Src. In co-infected combinations, the $p50^{cdc37}$/v-Src effect was synergistic (compare lanes 2, 5 and 6), but only modest cooperation was observed between $p50^{cdc37}$ and v-Ras (lanes 3 and 7). The cooperation of $p50^{cdc37}$ with v-Src and its dose dependent activation of Raf-1 is shown even more clearly in the dose response experiment shown in figure 4B.

Ser621 of Raf-1 is an indispensable major phosphorylation site, whose deletion (28), or substitution by either alanine or even with negatively charged aspartate inactivates the protein (20, 57), possibly by compromising the activation-competent conformation of the Raf-1 catalytic domain (55). Neither v-Src nor $p50^{cdc37}$ were capable of substantially inducing Raf-1(S621A) activation as compared with their strong positive effect on wild-type Raf-1 (fig. 4C, lanes 5 - 7) Interestingly, however, $p50^{cdc37}$ also enhances the weak effect of v-Src on the Raf-1 mutant as it does for wild-type Raf-1 (lanes 4 and 6). This result suggests that $p50^{cdc37}$, in conjunction with its partner Hsp90, may act as a chaperone by increasing the proportion of Raf-1 which is in the active conformation.

Inhibition of Raf-1 activation by dominant negative $p50^{cdc37}$ and GA. Since the deletion mutant $p50^{cdc37}\Delta C$ fails to bind to both mammalian and insect Hsp90, we sought to determine whether this mutant might interfere with Raf-1 activity by displacing the wild-type insect $p50^{cdc37}$/Hsp90 complex from Raf-1 since it retains the ability to bind to Raf-1 (fig. 2). In the detailed experiment shown in figure 5A, we attempted to correlate the effects of $p50^{cdc37}\Delta C$ on Raf-1 activity with its aforementioned ability to displace the full-length $p50^{cdc37}$ protein upon overexpression (fig. 2D). Previously it has been found that endogenous insect Hsp90 and $p50^{cdc37}$ associate with overexpressed mammalian Raf-1 in Sf9 cells(14, 15). However, since our $p50^{cdc37}$ antibody fails to recognize $p50^{cdc37}$ from insect cells, Sf9 cells were co-infected with baculoviruses expressing mammalian $p50^{cdc37}$ and Raf-1 alone or with increasing amounts of a baculovirus expressing $p50^{cdc37}\Delta C$. Extracts of infected cells were then immunoprecipitated with anti-Raf-1 and analyzed for associated mammalian $p50^{cdc37}$ proteins and HSP83, the endogenous

insect homologue of Hsp90 (11), as well as for Raf-1 kinase activity. Figure 5A shows, that as we had previously observed in mammalian cells (fig. 2C), $p50^{cdc37}\Delta C$ efficiently and in a dose-dependent manner displaced its full-length counterpart from Raf-1 in co-infected Sf9 cells and strongly reduced the association of insect Hsp90 with Raf-1. The dissociation of $p50^{cdc37}$ and Hsp90 from Raf-1 correlated closely with the reduction of Raf-1 activation to basal levels (fig. 5A, top). A control Western blot of total cellular extracts from this experiment indicates that this effect was not due to decreased expression of wild-type $p50^{cdc37}$, endogenous Hsp90, or Raf-1 kinase (fig. 5A, right panels). We conclude that $p50^{cdc37}\Delta C$ functions as a dominant negative for the $p50^{cdc37}$-mediated Raf-1/ $p50^{cdc37}$/Hsp90 complex formation and subsequent Raf-1 kinase activation.

We also examined whether $p50^{cdc37}\Delta C$ could inhibit Raf-1 activation by Ras and v-Src. Here again overexpression of $p50^{cdc37}\Delta C$ in insect cells abrogated Raf-1 activation by oncogenic Src and Ras (fig. 5Bi). Thus, both v-Src and v-Ras mediated activation of Raf-1 in Sf9 cells is dependent on the ability of $p50^{cdc37}$ and Hsp90 to form a productive complex with Raf-1 kinase. To gain more insight into the mechanism of $p50^{cdc37}$-dependent Raf-1 activation, we assessed the effects of wildtype and dominant negative $p50^{cdc37}$ on the activity of Raf-1 catalytic domain site mutants by co-infection of Sf9 cells. As expected, Raf-1(K375M), which is kinase inactive (17) could not be stimulated by $p50^{cdc37}$ or Src (not shown). Tyr340, and to a lesser extent Tyr341, have previously shown to be important regulatory sites, whose phosphorylation by tyrosine kinases presumably activates Raf-1 by interfering with negative regulation of the catalytic domain by the amino terminus of the protein (17). Since, as shown above, $p50^{cdc37}$ binds both *in vivo* and *in vitro* to the catalytic half of the Raf-1 protein and it interacts also both physically and functionally with Src kinases (6, 16 and data not shown), we reasoned that $p50^{cdc37}$'s role might be auxiliary to tyrosine kinase function, i.e. by facilitating or promoting Raf-1 tyrosine phosphorylation or by preserving the active Raf-1 conformation. To test this, we coexpressed in Sf9 cells $p50^{cdc37}$ along with Raf-1(Y340D), a constitutively active mutant (17). Indeed, $p50^{cdc37}$'s coexpression with Raf-1(Y340D) (fig. 5Bii), even at the highest possible amounts (not

shown), failed to further superinduce the already high basal activity of this mutant, consistent with the above hypothesized role for $p50^{cdc37}$. However, when we also tested the effect of $p50^{cdc37}\Delta C$ on Raf-1(Y340D), we found again the previously noted strong inhibition of Raf-1 activity (fig. 5Bii). Consistent with this, we have found that both $p50^{cdc37}$ and $p50^{cdc37}\Delta C$ associate with Raf-1(Y340D) as judged by examination of the co-expressed proteins (not shown). The above results argue strongly for a potential dual role of $p50^{cdc37}$ and its Hsp90 chaperone cofactor in the Raf-1 activation process. One where $p50^{cdc37}$/Hsp90 might be involved both in the efficient activation of Raf-1 and, a second in maintaining the active kinase conformation, once relief from repression by the N-terminal domain is achieved either through tyrosine phosphorylation by v-Src (fig. 4) or by activating amino acid mutations (fig. 5C).

Using a complementary experimental approach, we then tested whether GA-mediated inhibition of insect cell Hsp90 would abrogate baculoviral Raf-1 activation as we had observed in Cos-1 cells. Indeed, GA treatment of Sf9 cells co-infected with Raf-1 and viruses expressing v-Src, v-Ras, or $p50^{cdc37}$ resulted in dramatic decreases in Raf-1 activity (fig. 6A) that correlated with a substantial loss of endogenous Hsp90 binding to Raf-1 in all tested combinations (Figure 6B and data not shown). It is of note that under the conditions used, GA resulted in only slight depletion in Raf-1 protein, which, interestingly, exhibited a noticeable mobility up-shift during SDS-PAGE. Thus, the dramatic reduction in Raf-1 kinase activity cannot be accounted for by changes in levels of Raf-1 protein expression (control anti-Raf-1 immunoblot in fig. 6A). Thus, Raf-1 activation by co-expression with $p50^{cdc37}$, v-Src, or v-Ras is dependent in each case on functional endogenous insect Hsp90.

We then examined whether in a similar fashion, as previously found in Cos-1 cells, the GA inhibitory effect in Sf9 cells could be due to disruption of complex formation between Raf-1 and $p50^{cdc37}$/Hsp90. In agreement with both *in vitro* (fig. 1C) and *in vivo* reconstitution data from Cos-1 cells (fig. 2C), the experiment in figure 6B shows that co-expression of mammalian $p50^{cdc37}$ with Raf-1 in Sf9 cells results in strong $p50^{cdc37}$/Raf-1 complex formation and enhanced recruitment of endogenous Hsp90 into the kinase complex (compare lanes 1 and 3). This

correlates well with $p50^{cdc37}$-mediated Raf-1 activation as evidenced by the *in vitro* kinase activity of immunoprecipitated Raf-1 in a parallel assay (fig. 6B, top panel). However, in GA-treated replicate cultures, both these effects were almost entirely eliminated. We conclude, therefore, that under all conditions tested in both mammalian and insect cells, Raf-1 must be able to efficiently complex with both $p50^{cdc37}$ and Hsp90 in order to achieve and/or maintain its activated state.

$p50^{cdc37}$ contributes to the transduction of EGF signals that activate the MAPK cascade via Raf-1. Activated Raf-1 transduces signals to multiple pathways. The most well studied of these is the MAPK pathway. If, therefore, the association of the $p50^{cdc37}$/Hsp90 complex with Raf-1 contributes to the activation of Raf-1, the dominant negative mutant $p50^{cdc37}\Delta C$, which disrupts this complex, would be expected to interfere with the transduction of physiological signals from Raf-1 to the MAPK cascade. To test this hypothesis, we overexpressed $p50^{cdc37}\Delta C$ or its full-length $p50^{cdc37}$ counterpart in combination with Raf-1 and HA-Erk-1 in human embryonic kidney 293 cells. Serum-starved cultures were harvested with or without EGF stimulation and solubilized cell extracts were then examined by Western blotting with an antibody against activated phospho-MAPK or with control antibodies against transfected HA-Erk-1 or Raf-1 (fig. 7). The results revealed that in contrast to the wild-type protein (fig.7, lanes 4 and 5), $p50^{cdc37}\Delta C$ inhibited EGF stimulated Raf-1 activation as judged by Raf-1 kinase assay (not shown) and subsequent MAPK activation as determined by detection of dually phosphorylated HA-Erk-1 and endogenous Erk-2 with anti-phospho Erk antibodies (lanes 5 and 6). Thus, not only is the $p50^{cdc37}$ C-terminal mutant unable to support Raf-1 activation, but it also prevents Raf-1-mediated downstream signaling through the MAPK pathway. Thus both GA and $p50^{cdc37}\Delta C$, which target the same components of the Raf-1 activation complex, produce similar adverse effects: disruption of the native Raf-1 heterocomplex, inhibition of Raf-1 activation and, interruption of signaling to downstream Raf-1 effectors. These findings show that the

$p50^{cdc37}$/Hsp90 complex contributes to the activation of Raf-1 by EGF and plays a critical role in the transduction of EGF-generated Raf-1 signals to the MAPK pathway.

Expression of p50Cdc37 in MCF-7 cells. To determine if the effects of cdc37 on the MAP kinase pathway extend to breast cancer cells, we examined the role of cdc37 in the response to estrogen in the MCF-7 human breast cancer cell line. First we sought to determine whether $p50^{cdc37}$ is expressed in these cells. Previously it was reported that during lactation in mice there is a strong of regulation of $p50^{cdc37}$ in breast tissue in vivo(86). We have begun to determine whether this phenomenon can be recapitulated in breast cells in culture. To this end, MCF-7 human breast cancer cells were incubated in the presence of estrogen and various times after estrogen addition cell lysates were analyzed for the expression of cdc37 mRNA and protein. Results are shown in Fig. 8. Northern blot analysis indicates that there is constitutive expression of the CDC37 mRNA with a slight increase after estrogen addition. However, analysis of protein expression by Western blotting indicates that there is a several fold increase in p50cdc37 protein expression by 24 hours. Thus there may be post translational regulation of CDC37 expression in these cells. Also from these experiments, it appears that there is no gross structual alteration of the Cdc37 mRNA or protein.

The effect of CDC37 on estrogen receptor responses. Estrogen plays a key role in the regulation of breast cancer cell growth. The CDC37 partner Hsp90 has long been implicated in steroid receptor responses(67, 69). Recently, CDC37 has also been implicated in response of some steroid receptors(21). In addition, there is evidence that estrogen activates the Raf-1/MAP kinase pathway in these cells(50). Therefore, we sought to examine the effects of CDC37 on estrogen receptor activity.

For this purpose, we examined the response of the ERE-luceiferase reporter gene in the MCF-7 human breast cancer cell line. The results are shown in Fig. 9. In this cell line in the

absence of estrogen, $p50^{cdc37}$ has little or no effect on the expression of reporter gene. In the presence of estrogen, cotransfection of the $p50^{cdc37}$ gives a slight enhancement of expression of the reporter gene. In these cells, as expected, estrogen results in a significant 40 fold increase in the expression of reporter gene by itself. Interestingly, cotransfection of the dominant negative $p50^{cdc37}\Delta C$ in this cell line, has a dramatic effect on the response to estrogen. Though $p50^{cdc37}\Delta C$ had little effect by itself, it was able to decrease the response to estradiol by approximately tenfold. It should be noted that this experiment has only been performed once of the present time. We are currently in the process of reproducing this preliminary result. If it holds on, this will provide strong evidence that he CDC37 mediated process is required for estrogen receptor response in breast cancer cell lines.

To examine the effects of $p50^{cdc37}$ on estrogen regualtion of MAP kinase in MCF-7 cells, we have used a reporter gene assay that is sensitive and specific for the MAP kinase pathway. This assay uses the C-terminal domain of Elk-1 fused to a Gal4 DNA binding domain. Elk 1 binds to and is phosphorylated by MAP kinase. Upon phosphorylation, this fusion gene then transactivates a Gal4 UAS containing reporter gene(48). When the reporter gene and Gal4 Elk fusion are cotransfected into MCF-7 cells, robust activation of reporter gene is given by the addition of estradiol. (fig. 9B) This confirms the the MAP kinase pathway is activated by estradiol. Cotransfection in addition with wild type $p50^{cdc37}$ slightly increases the stimulation, although cotransfection of $p50^{cdc37}$ in the absence of estradiol has little effect. Strikingly, cotransfection of the dominant negative $p50^{cdc37}\Delta C$ almost entirely abolished activation of the MAP kinase specific reporter gene. This result indicates that estrogen dependent activation of MAP kinase in human MCF-7 cells requires Cdc37.

DISCUSSION

In the first year of this award, we have focused our efforts on understanding the role of the $p50^{cdc37}$/Hsp90 complex in the regulation of the Raf-1. This emphasis was justified by the importance of MAP kinase activation in estrogen growth regulation(7, 50) and the dramatic effects we have found that $p50^{cdc37}$ has on Raf-1 activation. We have found that coexpression of $p50^{cdc37}$ with Raf-1 leads to Raf-1 activation and that disruption of the cdc37/Hsp90 interaction with Raf-1 with either $p50^{cdc37}\Delta C$ or GA inhibits Raf-1 activation and signaling through Erk. These results indicate that the concerted action $p50^{cdc37}$ and Hsp90 plays a critical role in cell signaling via the Raf-1/Mek/Erk pathway including in the response to estrogen.

Recently, Perdew et al. (66) have found by immunoprecipitation and peptide mapping that cdc37 encodes the 50 kDa protein that previously has been described to associate with Hsp90 (65) and is found in both native Raf-1 and v-Src complexes (6, 84, 95, 97). Here we have expressed recombinant $p50^{cdc37}$ and directly examined its interactions with Hsp90 and Raf-1. We have found by all criteria that the recombinant $p50^{cdc37}$-reconstituted complexes are indistinguishable from those of pp50 described in the past (reviewed in Hunter and Poon (32) and Pratt and Toft (70)), not only in heterodimer formation with Hsp90, but also in higher-order heteroprotein complex formation involving Raf-1.

Moreover, we have explored the potential role of $p50^{cdc37}$ and its partner Hsp90 in the Raf-1 activation process. Our analysis indicates that Raf-1/Hsp90 association is for the most part $p50^{cdc37}$-dependent and that $p50^{cdc37}$ is the factor which primarily mediates the Raf-1/$p50^{cdc37}$/Hsp90 complex formation. More specifically, $p50^{cdc37}$ binds to the catalytic domain of Raf-1 through its N-terminus and tethers Hsp90 to Raf-1 through a second domain located at its C-terminal-half (see fig. 2D). This finding is consistent with Stepanova et al.'s(86) conclusion that $p50^{cdc37}$ accumulates Hsp90 to Cdk4. In addition, it validates experimentally the earlier proposal that Hsp90's specific associations might be mediated through Hsp90-associated cofactors and that pp50, in particular, might function in targeting Hsp90 to v-Src and Raf-1 kinases (9, 68, 69).

Several lines of evidence indicate that $p50^{cdc37}$/Hsp90 association with Raf-1 is necessary for the Raf-1 kinase activity. First, GA, an Hsp90-specific inhibitor, blunted Raf-1 activation by serum (fig. 3), and this inhibition correlated with a loss of $p50^{cdc37}$ and Hsp90 from the kinase without disrupting the $p50^{cdc37}$-Hsp90 association itself. The inhibition by GA was also observed with BXB-Raf-1, a constitutively active N-terminal Raf-1 deletion mutant (5), which consistently binds to $p50^{cdc37}$ and Hsp90 even more strongly than its full-length counterpart (data not shown). Coupled with our finding that $p50^{cdc37}$ brings Hsp90 into the Raf-1 complex, these results suggest that the interface of $p50^{cdc37}$-Raf-1 interaction is the target of GA action and that GA induced conformational alteration of the Hsp90/$p50^{cdc37}$ heterodimer leads either to the release of the heterodimer as a whole from Raf-1 or prevents it from rebinding to Raf-1. Freed Raf-1 then becomes subject to accelerated degradation as observed by Schulte et al. (77). That occupation of the ATP/ADP binding pocket by of Hsp90 by GA results in dissociation of the protein from Raf-1 is consistent with the notion that alternating cycles of ATP and ADP binding regulate Hsp90 conformation, and in turn its abilty to mediate the formation of productive signaling heterocomplexes (10, 26, 71, 90). Similarly, overexpressed $p50^{cdc37}\Delta C$ reduces both Hsp90 association with Raf-1 and Raf-1 kinase activity by competitively displacing wild-type $p50^{cdc37}$ from the Raf-1 complex. Mere addition of purified $p50^{cdc37}$ and HSP90 to Raf-1 does not activate the kinase in vitro (unpublished observation). Furthermore, it is worth noting that under commonly utilized kinase assay conditions, Raf-1, precipitated in RIPA buffer and thus presumably stripped of bound $p50^{cdc37}$ and Hsp90, is active. This suggests that $p50^{cdc37}$ and Hsp90 exert their activation role *in vivo* in conjunction with additional Raf-1 activation factors and do not need to stay associated with Raf-1 *in vitro* in order for the kinase to remain active. This also argues against a strictly structural role for the $p50^{cdc37}$/Hsp90 complex in Raf-1 activity.

Intriguingly, $p50^{cdc37}$ itself upon coinfection with Raf-1 results in strong Raf-1 stimulation. This activation is even stronger than that observed with v-Ras and only slightly weaker than v-Src mediated Raf-1 activation. Moreover, $p50^{cdc37}$ was able to weakly enhance v-Src-mediated activation of Raf-1(S621A), a Raf-1 kinase domain conformation-compromised and thus inactive

mutant. This suggests that $p50^{cdc37}$ may contribute to the formation or stabilization of the active Raf-1 conformational state and that as with v-Src and v-Ras it requires phosphorylatable Ser621 for function(57). In contrast, $p50^{cdc37}$ failed to induce further the already high constitutive activity of Raf-1(Y340D), an N-terminal repression-relieved activated Raf-1 mutant (17). One possible interpretation of this result is that $p50^{cdc37}$ might enhance Src-mediated phosphorylation and activation of Raf-1, a notion supported by the observed physical and functional interactions between Src kinases and $p50^{cdc37}$ (6 and unpublished results), including their strong synergistic effect on activating Raf-1 activation (fig. 4). However, our finding that the dominant negative $p50^{cdc37}$ deletion down-regulates Raf-1(Y340D) (fig. 5) in a dose-dependent fashion (not shown) indicates that some of the effects of $p50^{cdc37}$/Hsp90 complex are independent of tyrosine phosphorylation as well. Thus, it is likely that the $p50^{cdc37}$/Hsp90 complex is further required to maintain the activated Raf-1 kinase in its active conformation. This latter interpretation would be consistent as well with the findings that activated ras-independent Drosophila Raf alleles still require Hsp90 association for constitutive function at the membrane (93).

Previous work in Raf-1 overexpression systems has suggested that there may be a limiting cytosolic factor which is required for maximal Raf-1 activation(8, 30, 46, 88). Our results suggest that $p50^{cdc37}$ could well be a component of this activity. However, since $p50^{cdc37}$ is more abundant than Raf-1, the ability of $p50^{cdc37}$ overexpression to activate endogenous Raf-1 is modest relative to its marked ability to activate co-expressed Raf-1. This suggests that in unstimulated cells there may be a stoichiometric inhibitor of Raf-1 signaling whose effects are partially overcome by overexpression of Raf-1. Conceivably, under these conditions, the $p50^{cdc37}$/Hsp90 complex becomes limiting and thus stoichiometric overexpression of $p50^{cdc37}$ activates Raf-1. Serum stimulation of Raf-1 may have a comparable effect by allowing the $p50^{cdc37}$/Hsp90 complex to gain more efficient access to Raf-1.

A variety of evidence indicates that a principal function of Ras is to recruit Raf-1 to the plasma membrane(42, 87). Although not examined here directly, as previously noted for Hsp90(12, 39, 93), we have detected some plasma membrane localization of $p50^{cdc37}$ by

immunolocalization techniques and of Raf-1 associated $p50^{cdc37}$ and Hsp90 using cell fractionation (Grammatikakis, N., unpublished, and 6, 61, 68, 95). Although the membrane recruitment model of $p50^{cdc37}$/Hsp90 function cannot be ruled out , the finding in Drosophila that membrane bound Raf^{torY9} still requires Hsp90 for activity (93) also argues that possible membrane targeting of Raf-1 by the $p50^{cdc37}$/Hsp90 complex is at least not the only function of the complex.

Besides possibly promoting and maintaining active Raf-1 conformation as discussed above, additional roles for the $p50^{cdc37}$/Hsp90 heterodimer can be envisioned. The complex could serve to sterically hinder access of constitutive Raf-1 downregulators to Raf-1 or function as an adaptor or scaffolding protein for the Raf-1 activation complex. Consistent with the former possibility, Dent et al.(14, 15) and Jelinek et al. (34) have found that *in vitro* Ras-GTP activated Raf-1 is rapidly inhibited by unidentified accumulated phosphatases, a process that can be partially reversed by the addition of purified Hsp90. Also consistent with this model is the observation that $p50^{cdc37}$ competes with p60/Hop and PP5 phosphatase for Hsp90 binding(61, 81, Grammatikakis, N, unpublished). In contrast to the positive effects of $p50^{cdc37}$ on Raf-1 activity, p60/Hop has been found to be associated with GA-inhibited inactive steroid receptor(83) and Raf-1 complexes(68). This also suggests that the concerted action of additional chaperones and co-factors, including Hsp70 and p60/Hop, may play a role in the regulation of kinases by $p50^{cdc37}$ and Hsp90.

With regard to the second model, synthetic oligomerization of Raf-1 has been reported to activate Raf-1 through both Ras independent (18) and Ras dependent (45) mechanisms. Hsp90 has a long recognized intrinsic capacity to both form homodimers (51, 52, 59) and also to oligomerize (60). Therefore, it is possible that $p50^{cdc37}$/Hsp90 complexes might serve as a structural and functional scaffold on which Raf-1 oligomerization and activation take place (see fig. 2D). This is an interesting possibility, especially in the context of a dynamic signalsome (63) that includes Mek and Erk kinases along with Ksr-1 and 14-3-3 (40 , 92, this report).

Consistent with the above possibility, we have found that $p50^{cdc37}$ forms a stable complex with Mek-1, the direct Raf-1 substrate. This could be significant for signal propagation within the MAPK module. In this case, in sharp contrast with other $p50^{cdc37}$ heteroprotein complexes,

including with Raf-1 (this work), v-Src (6) and Cdk4 (24, 86 and Grammatikakis et al., in preparation), the Mek-1/p50^{cdc37} complex is devoid of Hsp90. The functional significance of this is unclear, but since the bulk of p50^{cdc37} is sequestered by Hsp90 (97 and Figures 1A and 3), it implies that Mek-1 may compete with Hsp90 for binding to the same domain on p50^{cdc37}. In addition, we have noticed that the level of Mek-1 association with Hsp90-free p50^{cdc37} is inversely proportional to Raf-1 activity after serum stimulation but increases during serum deprivation or GA treatment when, notably, Raf-1 is least well bound with p50^{cdc37} and inactive (fig. 3 and not shown). Thus, it is possible that in the absence of growth factors, Mek-1 prevents the p50^{cdc37}/Hsp90 complex from associating with Raf-1 and thereby downregulates its activity. Indeed, a distinct attenuation effect by Mek-1 on Raf-1 activity has already been described, which may occur either through triggering inhibitory autophosphorylation of Raf-1(88) or by downstream kinases which directly phosphorylate and downregulate Raf-1(1, 2, 74, 91, 96). This possibility is further suggested by our observation that these interactions might be regulated by the phosphorylation and activation status of Raf-1 since, p50^{cdc37} and Mek-1 preferentially associate with distinct populations of Raf-1, i.e. hypo- and hyperphosphorylated forms, respectively. Determining the precise roles of p50^{cdc37} and Mek-1 during Raf-1 signaling is subject of ongoing investigation.

An important remaining question is whether the associations or the activity of the p50^{cdc37}/Hsp90 complex are subject to regulation. We have found increased formation of the Raf-1/p50^{cdc37}/Hsp90 ternary complex after serum stimulation. It is possible that this contributes to the activation of Raf-1. This would be consistent as well with our finding that co-expression of p50^{cdc37} with Raf-1 accumulates Hsp90 and activates Raf-1 in a dose dependent manner. Analogously, Garcia-Cardena et al.(22) have recently found that extracellular regulators of endothelial nitric oxide synthase induce the rapid recruitment of Hsp90 to eNOS which results in the membrane activation of this enzyme. Moreover, since both p50^{cdc37} and Hsp90 are phosphoproteins (Figure 3, and refs(6, 44, 95, 97)), their respective protein associations could in turn be modulated by phosphorylation. In addition, serum regulation of the phosphorylation state

of the Hsp90/p50^{cdc37} complex could play an important role in Raf-1 activation. Alternatively, serum might regulate the nucleotide binding state and conformation of Hsp90 (26, 71) that is associated with p50^{cdc37} and Raf-1 and thereby allosterically regulate its effects on Raf-1. This may occur either through assisting Raf-1 in the conformational transition to the activated state or by allowing it to achieve a configuration where it is competent to respond to upstream activators.

We have also examined the role of p50^{cdc37} in the response of MCF-7 cells to estrogen. We have found that dominant negative p50$^{cdc37}\Delta$C can inhibit both estrogen dependent activation of the MAP kinase pathway and estrogen mediated activation of an ERE reporter gene. Estrogen is thought to activate the MAP kinase pathway in through effects on the c-src kinase(50). Src alone can activate Raf and can synergize with Ras for Raf activation. Thus the inhibition of MAP kinase activation in response to estrogen in MCF-7 cells would be consistent with our findings that dominant negative p50$^{cdc37}\Delta$C can inhibit Raf-1 activation which is downstream of Src.

The mechanism by which dominant negative p50^{cdc37} may inhibit estrogen mediated transcription is less clear. This result may indicate a direct role for p50^{cdc37} in estrogen receptor function. Certainly HSP90 is required for the function of some steroid receptors including the estrogen receptor(3, 68, 73) and yeast p50^{cdc37} is required for the function of androgen receptors in yeast(21). Though there is evidence that Hsp90 interacts with the estrogen receptor, is unclear whether mammalian p50^{cdc37} does so as well. Future work will be necessary to directly test this possibility.

An alternate mechanism for inhibition of estrogen receptor function by dominant negative p50^{cdc37} could be that these effects are mediated indirectly by the effects of p50^{cdc37} on MAP kinase. Not only can estrogen activate MAP kinase, but MAP kinase activation can in some case activate the estrogen receptor(37). If phosphorylation of the estrogen receptor by MAP kinase plays a role in estrogen dependent transcription in MCF-7 cells, then dominant negative p50^{cdc37} may be inhibiting the transcriptional effects of the estrogen receptor in directly by inhibiting its phosphorylation by MAP kinase. Future experiments with dominant negative MAP kinase constructs should discriminate between these two possibilities.

CONCLUSIONS

In summary, our findings complement genetic data from yeast and Drosophila and indicate that the $p50^{cdc37}$/Hsp90 chaperone complex is essential for Raf-1 activation and signaling through the MAPK pathway. Interestingly, the fact that Raf-1 (89, 94), Hsp90 (70) and $p50^{cdc37}$ (11 and Grammatikakis, in preparation) are all ubiquitously expressed points to a potentially universal Raf-1/Hsp90/$p50^{cdc37}$ signaling complex. Our results furthermore indicate that $p50^{cdc37}$ plays a crucial role in function of estrogen in MCF-7 cells. These results furthermore indicate that drugs that would inhibit $p50^{cdc37}$ function could potentially be useful in the treatment of breast cancer.

A note regarding the Statement of Work

The order in which we have approached this project differs somewhat for that described in the Statement of Work in the orginal application. Largely as a result of our exciting findings regarding the role of $p50^{cdc37}$ in Raf-1 regulation, we have focussed on Technical Objective 2. The tasks which this work corresponds to are Task numbers 5, 7, 8, 10, 13, and 15 in the original proposal. In the upcoming year more emphasis will be placed on Technical Objectives 1 and 3 in the original proposal.

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FIGURE LEGENDS

FIG. 1. Association of $p50^{cdc37}$, Hsp90 and Raf-1 in vivo and in vitro.

A. Anti-Raf-1 immunoprecipitation (IP) from ^{35}S-methionine labeled Cos-1 cells (lane 1). Lanes 2-5: After the primary anti-Raf IP was boiled for 2 min in the presence of 0.5% SDS, a second IP was carried out with anti-Hsp90 or control antibody (lanes 2 and 3) or with polyclonal anti-$p50^{cdc37}$ or non-immune rabbit antibody (lanes 4 and 5, respectively). Lanes 6 and 7: Anti-$p50^{cdc37}$ primary IPs and non-immune rabbit serum IPs form ^{35}S-methionine labeled Cos-1 cells, respectively. A second IP with anti-Hsp90 antibody (lane 8) was performed using an identical fraction of the anti-$p50^{cdc37}$ primary immunoprecipitate run in lane 6. The relative migration of molecular weight marker proteins is indicated.

B. pMT3-HA-$p50^{cdc37}$ or pMT3-HA vector plasmids were transiently transfected into Cos-1 cells and extracts were immunoprecipitated with anti-FLAG/M5 as a control (lane 1) or anti-HA/12CA5 monoclonal under either denaturing or mild conditions (RIPA or NP40-LB buffer, lanes 2 and 3, respectively) or, to purify endogenous Raf-1 and $p50^{cdc37}$ proteins, with anti-Raf-1 (lane 4) or anti-$p50^{cdc37}$ (lane 5) monoclonal antibodies. Immunoprecipitated proteins were examined by Western blot/ECL for the presence of transfected HA-$p50^{cdc37}$ or endogenous $p50^{cdc37}$ with anti-HA antibody or anti-$p50^{cdc37}$ rabbit antisera and for endogenous Hsp90 or Raf-1 proteins with rat-anti-Hsp90 or rabbit-anti-Raf-1 antibodies.

C. FLAG-$p50^{cdc37}$ (purified from baculovirus infected Sf9 cells) or Hsp90 (purified from bovine brain; Stressgen) were assayed in vitro for binding to bacterially produced GST-Raf-1, GST-$p50^{cdc37}$, or GST alone as indicated by GSH-sepharose pull down and Western blotting with the indicated antibodies as described in the Materials and Methods section. Anti-Hsp90 immunoblotting performed with two distinct Hsp90-specific antibodies (SPA-830 and SPA-771) is shown (bottom two panels). The first two lanes indicate the input amounts of purified proteins added.

FIG. 2. The N-terminal half of $p50^{cdc37}$ mediates association with the catalytic domain of Raf-1, but is impaired for Hsp90 interaction and accumulation to Raf-1.

A. pSG5-$p50^{cdc37}$ or pSG5-$p50^{cdc37}\Delta C$ plasmids were transcribed and translated in vitro using T7 RNA polymerase and a reticulocyte lysate system (Promega). 5 µl of each reaction was either analyzed directly (input lanes) or assayed in vitro for binding to either GST or bacterially purified GST-ΔN-Raf-1(Δ26-309) and visualized by SDS-PAGE and fluorography. Comparable results were obtained with full length GST-Raf-1 (not shown).

B. pSG5-FLAG vector, pSG5-FLAG-$p50^{cdc37}$, or pSG5-FLAG-$p50^{cdc37}\Delta C$ transfected Cos-1 cells were ^{35}S-methionine labeled and anti-FLAG IPs in NP40 LB of each transfected sample were analyzed by SDS-PAGE and fluorography (lanes 1, 2, and 3, respectively). Proteins at the sizes predicted for overexpressed FLAG-$p50^{cdc37}$ proteins or associated endogenous Hsp90 are identified with open and closed arrows, respectively.

C. 2 µg pEBG-GST-Raf-1 was cotransfected with 5 µg pSG5-FLAG vector (lane 1), pSG5-FLAG-$p50^{cdc37}$ or pSG5-FLAG-$p50^{cdc37}\Delta C$, at 5 or 15 µg as indicated (lanes 2,3 and 4,5, respectively). After 48 hrs in 10% FBS in DMEM, all five cultures were harvested and lysed in NP-40 LB and GST-Raf-1 was GSH-sepharose purified and tested for associated $p50^{cdc37}$ or Hsp90 proteins with rabbit anti-$p50^{cdc37}$ or rat anti-Hsp90 antibodies. A control anti-GST immunoblot was also included to detect GST-Raf-1 (top panel).

D. Diagram indicating regions of interaction between $p50^{cdc37}$, Raf-1, and Hsp90.

The N-terminal half of $p50^{cdc37}$ (gray area) which corresponds to $p50^{cdc37}\Delta C$ is sufficient for interacting with the C-terminal kinase domain of Raf-1 while its C-terminal half mediates Hsp90 interaction (indicated by the heavy arrows). A distinct weak interaction of Raf-1 directly with Hsp90 through as yet unidentified domains is also proposed and is indicated by the gray arrow. The relative positions of the Y340 and S621 phosphorylation sites present on Raf-1 are also indicated. Since Hsp90 can both homodimerize and form oligomers through its C-terminus (DM/OM) (52, 59, 60), higher order complexes of $p50^{cdc37}$/Raf-1/Hsp90 can also be envisioned.

FIG. 3. Association of $p50^{cdc37}$ and Hsp90 with Raf-1 correlates closely with Raf-1 kinase activity. 2 μg each of pEBG-GST-Raf, pEBG-$p50^{cdc37}$, or pEBG-Mek-1 were transfected into subconfluent Cos-1 cells and next day each of the transfected 150 mm plates were further split into three 100 mm plates. 16 hrs later, cultures were fed with serum-free media for an additional 16 hrs. GA or only DMSO diluent was then added, followed by serum stimulation as indicated, and the three replicate cultures of each transfection were harvested and solubilized in NP-40 LB. GST-fusion proteins were then purified by GSH-affinity chromatography as described in the Materials and Methods section and analyzed for associated proteins by SDS-PAGE and immunoblotting with the indicated antibodies. 0.2x extract portions were similarly processed and tested for GST-Raf-1 or GST-Mek-1 kinase activity towards recombinant kinase-defective (KD) Mek-1, Erk-1, respectively, or no substrate as indicated for GST-$p50^{cdc37}$ associated kinase activity (top panels). Bottom panels: control immunoblots of total cell extracts. Control transfections with empty pEBG vector, followed by GSH pull-downs/Western blotting, showed that no $p50^{cdc37}$, Hsp90 or Raf-1 associated with the GST propeptide alone (not shown). Arrows in anti-Raf-1 immunoblots denote the bulk of fast and slower migrating Raf-1 which copurifies with GST-$p50^{cdc37}$ and GST-Mek-1, respectively.

FIG. 4. Sf9 cell coinfection with $p50^{cdc37}$ results in Raf-1 activation.

A. Baculoviruses encoding Raf-1, v-Src, v-Ras or $p50^{cdc37}$ were infected in Sf9 cells in the combinations indicated and 48 hrs post-infection, Raf-1 was immunoprecipitated with anti-Raf-1 polyclonal antibody (C-12) in RIPA buffer and tested for its ability to phosphorylate recombinant kinase-defective Mek-1, as described in the Materials and Methods section (top). Kinase assay reactions were also immunoblotted with the same anti-Raf-1 antibody (bottom).

B. Baculovirus co-infection followed by Raf-1 kinase assay and Western blot were performed as in A. In each set, increasing amounts of $p50^{cdc37}$ baculovirus (at 1, 3 and 9X) were added as indicated.

C. Wild-type Raf-1 or mutant Raf-1 containing a serine-to-alanine substitution at amino acid position 621 (Raf-1(S621A)) were either expressed alone or coexpressed with indicated v-Src or $p50^{cdc37}$ baculovirus constructs, immunoprecipitated and assayed for in vitro kinase activity as in A.

FIG. 5. $p50^{cdc37}\Delta C$ disrupts Raf-1/$p50^{cdc37}$/Hsp90 complex formation and abrogates Raf-1 activation.

A. A baculovirus encoding $p50^{cdc37}\Delta C$ mutant was co-infected in the same or in triple excess m.o.i. with $p50^{cdc37}$ (lanes 4 and 5) and Raf-1. Control Sf9 cultures included an empty-vector baculovirus infection (lane 1) and cultures infected with Raf-1 alone or in combination with $p50^{cdc37}$ (lanes 2 and 3, respectively). 48 hrs post-infection, cells were solubilized in NP-40 LB and portions of each of the five extracted cultures was harvested, subjected to anti-Raf-1 IPs under non-denaturing conditions using NP-40 LB lysis buffer (see Methods section) and analyzed either for Raf-1 kinase activity toward inactive recombinant Mek-1 (top), or for $p50^{cdc37}$ and Hsp90 associated proteins. For assessment of protein expression, control immunoblots of total cellular extracts are also presented on the right.

B. $p50^{cdc37}\Delta C$ inhibits v-Src, v-Ras, and $p50^{cdc37}$ mediated Raf-1 activation.

i. Raf-1 was immunoprecipitated and analyzed for its activity towards recombinant inactive Mek-1 from Sf9 cells co-infected with the indicated baculoviruses as described in FIG. 4A. The effect of v-Src (lanes 6 and 7) was examined in a separate experiment and involves a shorter kinase assay exposure.

ii. The effect of $p50^{cdc37}\Delta C$ on the constitutively active Raf-1(Y340D) mutant was examined as above. For comparison, wildtype Raf-1 was subjected to similar analysis and is shown in lane 4.

FIG. 6. GA inhibits Raf-1 activation in Sf9 cell by disrupting Raf-1/Hsp90/p50^{cdc37} complex formation.

A. Raf-1 alone or in combination with v-Src, v-Ras or p50^{cdc37} was expressed in Sf9 cells and following a 48 h incubation was immunoprecipitated with anti-Raf-1 polyclonal antisera in RIPA buffer and tested for in vitro kinase activity. Even-numbered lanes represent parallel cultures treated with GA (2 ug/ml) for 4 hrs before similarly harvested and analyzed. Blotted kinase reactions (top panel) were tested for immunoprecipitated Raf-1 protein levels using rabbit anti-Raf-1 immunoblotting (bottom). Note that GA-treated Raf-1 migrates slower than non-treated samples (bottom) and is severely deficient in phosphorylating recombinant kinase-inactive Mek-1(top panel).

B. Sf9 cell cultures co-infected with Raf-1 and p50^{cdc37} or empty-vector baculovirus were each split into two replicate cultures 24 hrs post-infection. 24 hrs later, one replicate culture was treated with GA (2ug/ml) for 2 hrs while the other was similarly treated with only DMSO diluent as indicated. Cell extracts in NP40 LB were subjected to Raf-1 IP followed by Raf-1 kinase assay (top panel) or Western blot analysis (bottom, left) or, additionally, directly analyzed for respective Raf-1, p50^{cdc37} or Hsp90 protein expression (C is as in lane 3 but immunoprecipitating Raf-1 antibody was omitted.) Open arrowheads denote position of immunoprecipitating anti-Raf-1 antibodies.

FIG. 7. Dominant negative p50^{cdc37} inhibits MAPK activation.

Duplicate 293 human embryonic kidney cells transiently transfected with pMT2-Raf-1, pMT2-HA Erk-1 and p50^{cdc37}, or p50$^{cdc37}\Delta$C, or vector alone were serum-starved and EGF stimulated. Solubilized extracts were then analyzed both with anti-active-Erk rabbit anti-serum (bottom) or for levels of expression with anti-HA or anti-Raf-1 antibodies (top two panels). Besides activated HA-Erk-1, 293 cells express both Erk-1 and Erk-2 isoforms which are also recognized by the anti-active-Erk polyclonal antiserum. Note that supershifted Raf-1 correlates closely with activated Erk contained in the same samples.

Fig.8. Expression of CDC37 mRNA and protein in MCF-7 cells.

A. Confluent MCF-7 cells were serum starved overnight in serum-free/phenol-red free DMEM and treated with 10 nM estradiol for an indicated amount of time. Total cell RNA was analyzed by Northern. For Cdc37, a internal coding sequence of cdc37 (SmaI fragments) were used as probes.

B. Similar as in A, but cells were directly lysed in SDS-PAGE sample buffer and then processed for Western Blotting using the anti-$p50^{cdc37}$ antibody.

Fig. 9. Inhibition of estogen action in MCF-7 cells by dominant negative Cdc37.

A. MCF-7 cells in phenol-free DMEM were transfected with 50 ng ERE-luciferase and the indicated $p50^{cdc37}$ expression plasmid per well (24-well plate) using Fugene-6 (Boerhinger-Mannheim). Next day, estrogen was added, and cell were incubated for 24 additional hours until lysis and measurement of luciferase activity. TK-Renillin luciferase activity was used as transfection efficiency control.

B. Similar to A except that the detection system used is the Stratagene Path Detect system for Elk1. This consists of a Gal4 DNA binding domain fused to the Elk-1 C-terminal activation domain and a Gal4 UAS driving a luciferase reporter gene. DNcdc37 is the dominant negative $p50^{cdc37}\Delta C$.

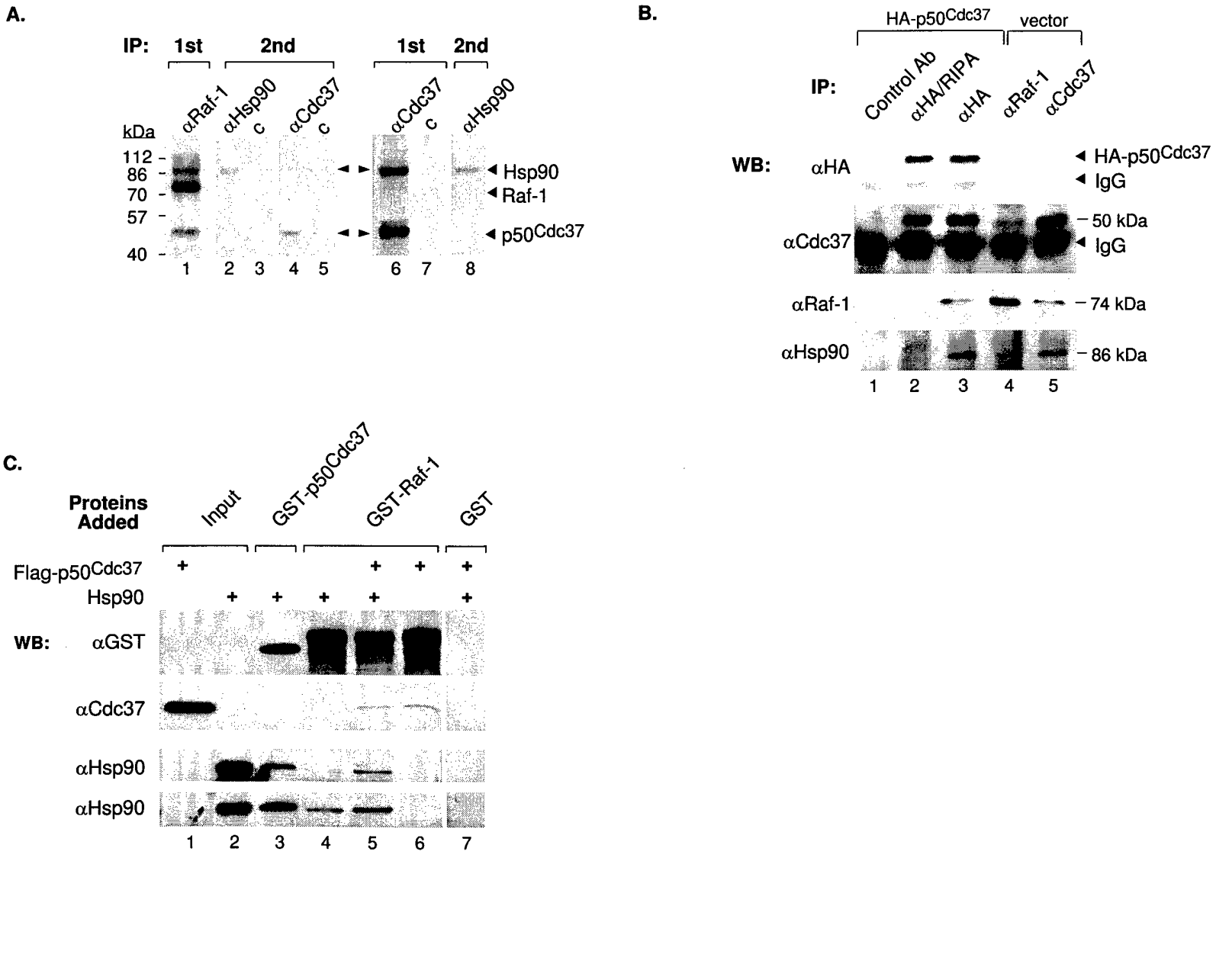

A.
IP:
1st
2nd
1st
2nd
αRaf-1
αHsp90
αCdc37
αCdc37
αHsp90
kDa
112
86
70
57
40
Hsp90
Raf-1
p50Cdc37
1 2 3 4 5 6 7 8
B.
HA-p50Cdc37
vector
IP:
Control Ab
αHA/RIPA
αHA
αRaf-1
αCdc37
WB:
αHA
HA-p50Cdc37
IgG
αCdc37
50 kDa
IgG
αRaf-1
74 kDa
αHsp90
86 kDa
1 2 3 4 5
C.
Proteins Added
Input
GST-p50Cdc37
GST-Raf-1
GST
Flag-p50Cdc37
Hsp90
WB:
αGST
αCdc37
αHsp90
αHsp90
1 2 3 4 5 6 7

Fig. 1

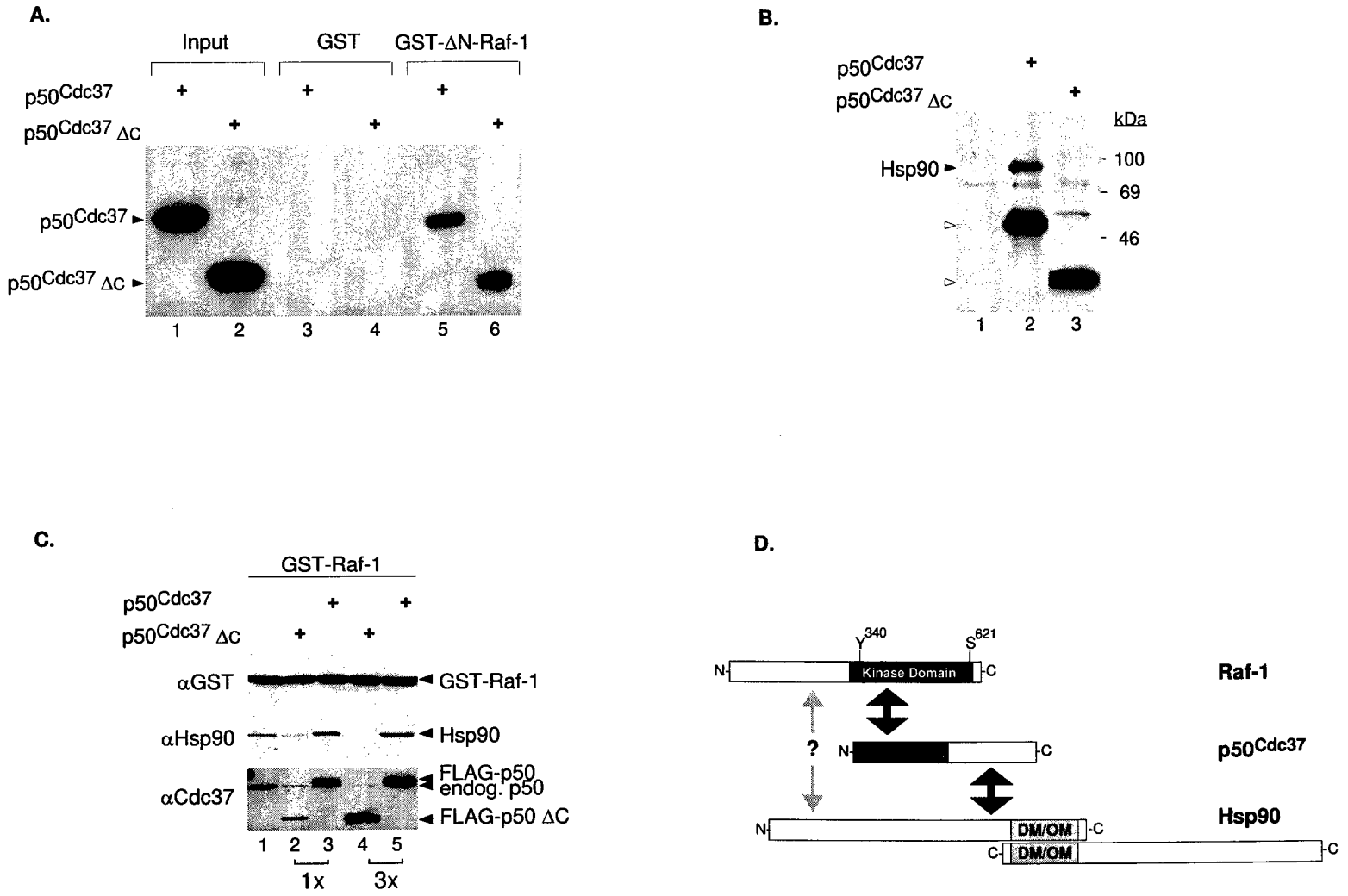

A.
Input
GST
GST-ΔN-Raf-1
p50Cdc37
p50Cdc37 ΔC
p50Cdc37
p50Cdc37 ΔC
1 2 3 4 5 6
B.
p50Cdc37
p50Cdc37 ΔC
kDa
Hsp90
100
69
46
1 2 3
C.
GST-Raf-1
p50Cdc37
p50Cdc37 ΔC
αGST
GST-Raf-1
αHsp90
Hsp90
αCdc37
FLAG-p50
endog. p50
FLAG-p50 ΔC
1 2 3 4 5
1x
3x
D.
Y340
S621
N
Kinase Domain
C
Raf-1
?
p50Cdc37
Hsp90
DM/OM
DM/OM

Fig. 2

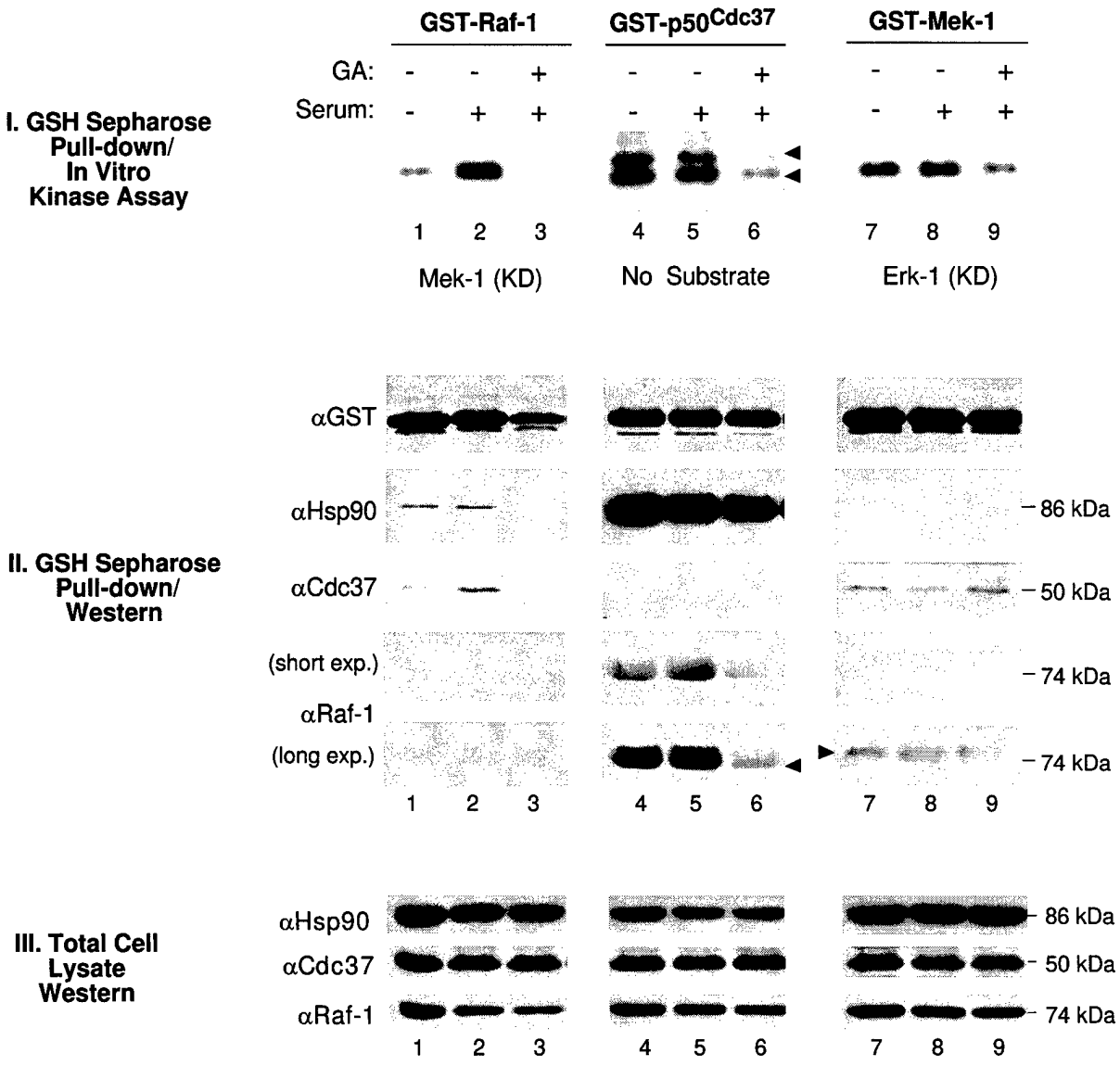
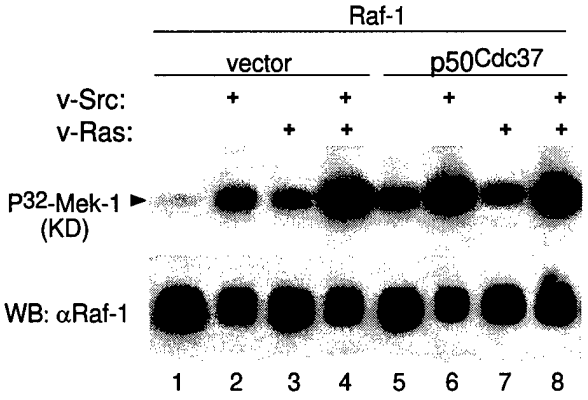
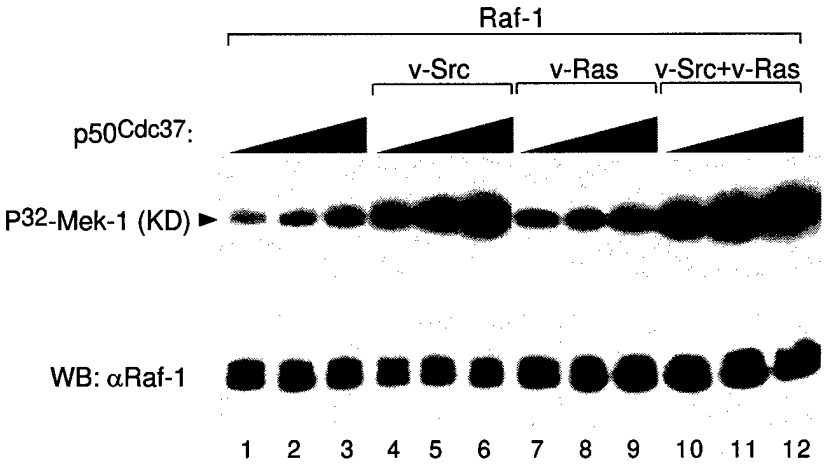

GST-Raf-1
GST-p50Cdc37
GST-Mek-1
GA:
Serum:
I. GSH Sepharose Pull-down/ In Vitro Kinase Assay
1 2 3 4 5 6 7 8 9
Mek-1 (KD)
No Substrate
Erk-1 (KD)
αGST
αHsp90
αCdc37
(short exp.)
αRaf-1
(long exp.)
II. GSH Sepharose Pull-down/ Western
86 kDa
50 kDa
74 kDa
74 kDa
III. Total Cell Lysate Western
αHsp90
αCdc37
αRaf-1
86 kDa
50 kDa
74 kDa

Fig. 3

A.


Raf-1
vector
p50Cdc37
v-Src:
v-Ras:
P32-Mek-1 (KD)
WB: αRaf-1
1 2 3 4 5 6 7 8

B.


Raf-1
v-Src
v-Ras
v-Src+v-Ras
p50Cdc37:
P32-Mek-1 (KD)
WB: αRaf-1
1 2 3 4 5 6 7 8 9 10 11 12

C.

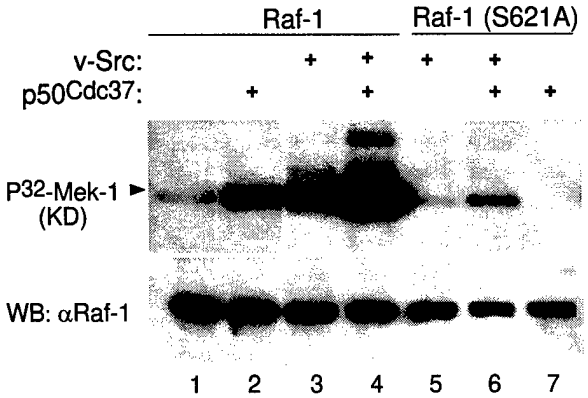

Raf-1
Raf-1 (S621A)
v-Src:
p50Cdc37:
P32-Mek-1 (KD)
WB: αRaf-1
1 2 3 4 5 6 7

Fig. 4

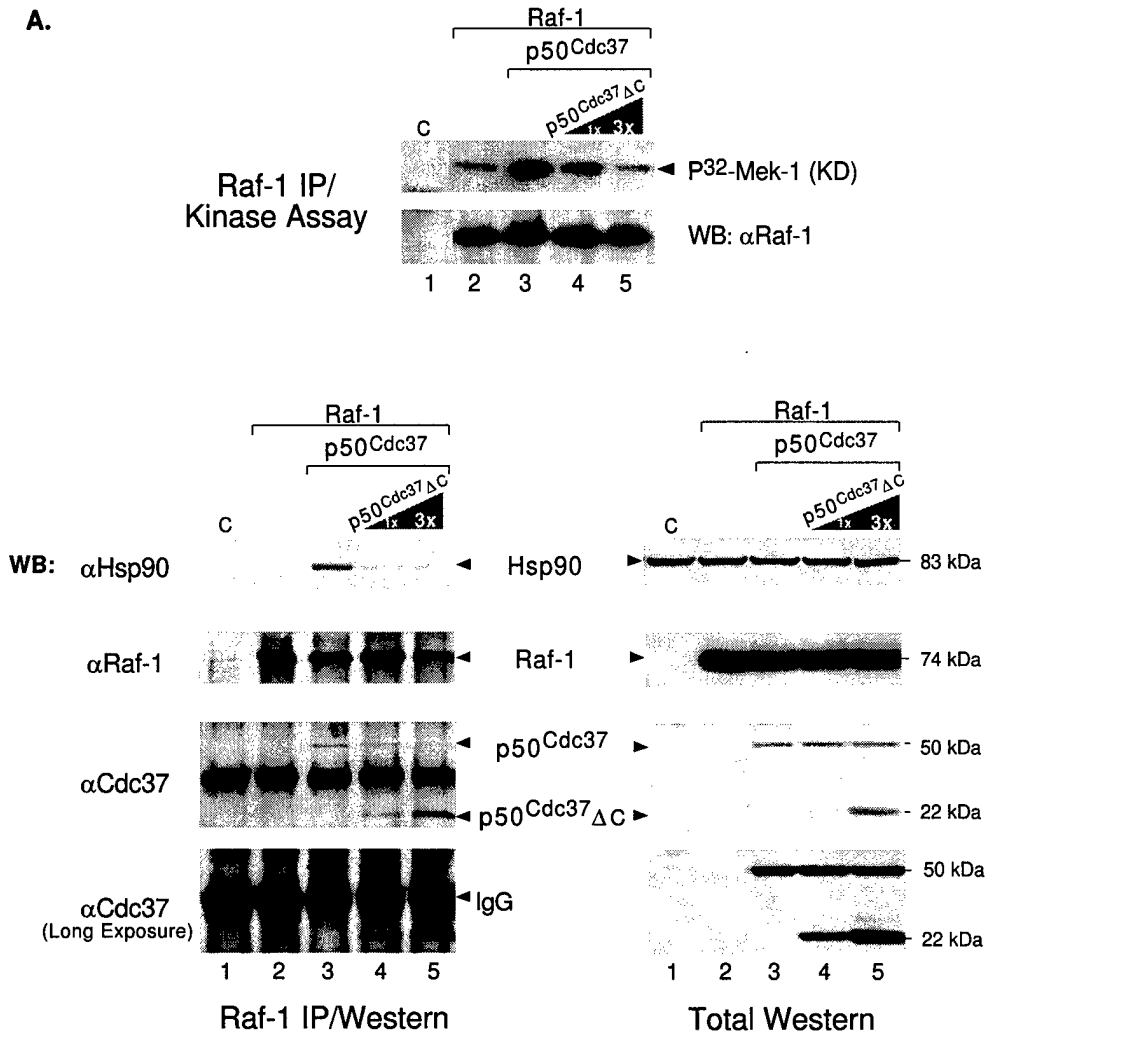
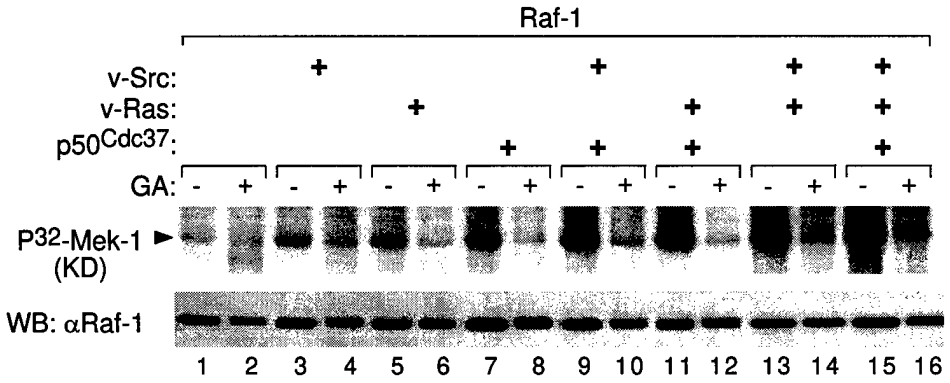

A.
Raf-1
p50Cdc37
p50Cdc37 ΔC
C
1x 3x
Raf-1 IP/
Kinase Assay
P32-Mek-1 (KD)
WB: αRaf-1
1 2 3 4 5
Raf-1
p50Cdc37
p50Cdc37 ΔC
C
1x 3x
WB: αHsp90
αRaf-1
αCdc37
αCdc37
(Long Exposure)
IgG
1 2 3 4 5
Raf-1 IP/Western
Raf-1
p50Cdc37
p50Cdc37 ΔC
C
1x 3x
Hsp90
Raf-1
p50Cdc37
p50Cdc37 ΔC
83 kDa
74 kDa
50 kDa
22 kDa
50 kDa
22 kDa
1 2 3 4 5
Total Western
B.
I.
Raf-1
v-Ras
p50Cdc37
v-Src
p50Cdc37 ΔC: + + +
P32-Mek-1 (KD)
WB: αRaf-1
1 2 3 4 5 6 7
II.
Raf-1(Y340D)
Raf-1
p50Cdc37 : +
p50Cdc37 ΔC: +
P32-Mek-1
(KD)
WB: αRaf-1
1 2 3 4

Fig. 5

A.


Raf-1
v-Src:
v-Ras:
p50Cdc37:
GA:
P32-Mek-1 (KD)
WB: αRaf-1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

B.

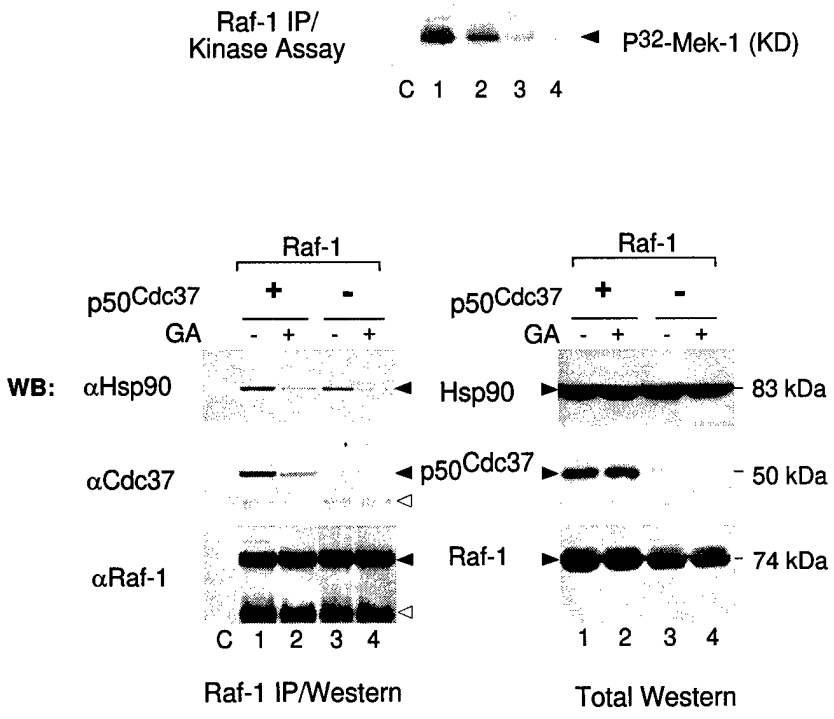
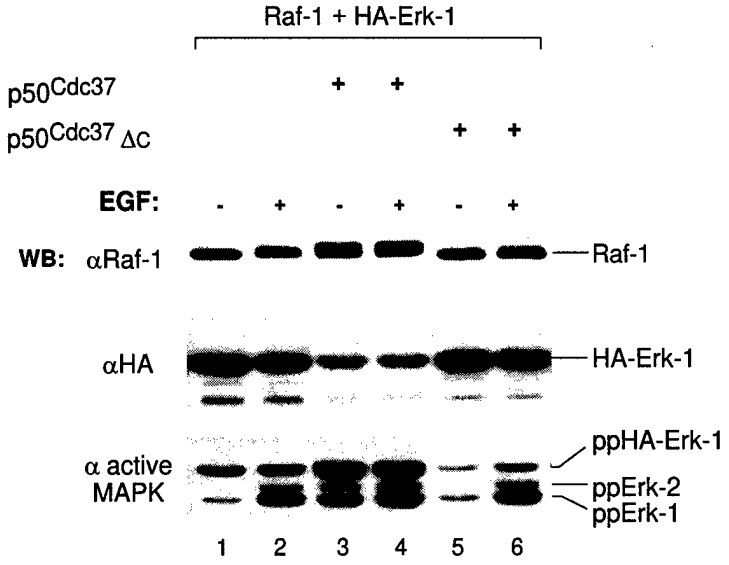

Raf-1 IP/ Kinase Assay
P32-Mek-1 (KD)
C 1 2 3 4
Raf-1
p50Cdc37
GA
WB:
αHsp90
αCdc37
αRaf-1
Hsp90
p50Cdc37
Raf-1
83 kDa
50 kDa
74 kDa
Raf-1 IP/Western
Total Western

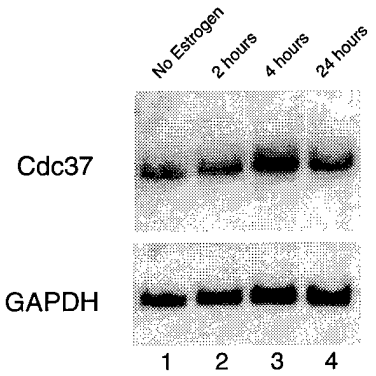
Fig. 6



Whole Cell Extract Western

Fig. 7

A. Northern Blot



B. Western Blot

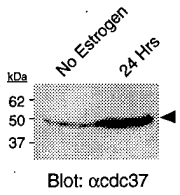


Fig. 8

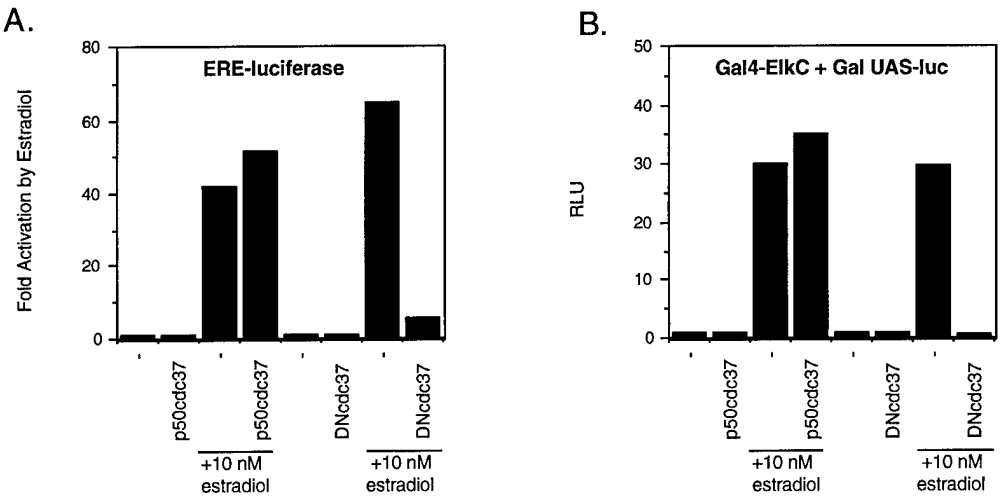

A.
ERE-luciferase
Fold Activation by Estradiol
80
60
40
20
0
-
p50cdc37
-
p50cdc37
-
DNcdc37
-
DNcdc37
+10 nM estradiol
+10 nM estradiol
B.
Gal4-ElkC + Gal UAS-luc
RLU
50
40
30
20
10
0
-
p50cdc37
-
p50cdc37
-
DNcdc37
-
DNcdc37
+10 nM estradiol
+10 nM estradiol

Fig. 9.